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The common Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* produce toxins, such as SEB, that cause diseases ranging from food poisoning to an acute, life-threatening toxic-shock syndrome. Vaccines for this and other related toxins are currently in the advanced development stage. Toxic-shock syndrome is predominantly diagnosed in women, and has been linked to toxin production from bacteria colonizing tampons. Over-stimulation of the immune response is responsible for the acute pathological effects and recovery is often followed by a profound nonresponsiveness to the toxin. The residual effects of suppressed toxin-specific immune responses may hinder successful vaccination of affected women. The results of our study of active duty military personnel between the ages of 18 and 35 years of age suggests a possible discordance between males and females in preexisting immune responses to the toxins, and further indicate the possibility of vaccination failures for women as a result of T-cell anergy.

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Robert S Wood 15 Jan 97

PI - Signature Date

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INTRODUCTION

The incapacitating and potentially lethal effects of toxic-shock syndrome are a significant health concern for women in military service and within the general population. Greater than 95% of reported cases occur in women (Center for Disease Control, 1982) and this disease has been linked to use of tampons. Toxic shock syndrome is caused by staphylococcal enterotoxins (SEs), toxic shock syndrome toxin (TSST-1) and certain other related proteins which are produced by *S. aureus* and *S. pyogenes* (Vergeront et al. 1983; Stolz et al. 1985; Crass and Bergdoll, 1986). Collectively these exotoxins are referred to as superantigens (SAgs), because of their profound effects upon the immune system. Most commonly the SEs are a cause of acute, self-limiting, food poisoning. The more severe consequences result from exposure to these toxins through a nonenteric route. Toxic-shock syndrome is the most frequent nonenteric disease that is associated with SAgs.

A more complete understanding of the diseases caused by SAgs is warranted because vaccination of combat troops has been a proposed countermeasure (unpublished). Both T-cell immunity and serum antibody levels are indicators of prior exposure to SAgs and these data are critical because one of the secondary effects of SEB and related toxins is to specifically inactivate T cells, thereby circumventing an appropriate immune response. At present there is a lack of information concerning any gender-based differences within military personnel in levels of circulating antibodies and T cell responses to bacterial SAgs.

There is extensive clinical documentation of a toxic shock syndrome that is caused by both staphylococcal and streptococcal SAgs (Stolz et al. 1985; Crass and Bergdoll, 1986; Schlievert et al., 1993). The symptoms produced by each SAg are similar and characterized by a rapid drop in blood pressure, elevated temperature, rash followed by a desquamation of the palms of the hands and multiple organ failure (Freedman and Beer, 1991). There are additional environmental factors, such as endotoxin from Gram-negative bacteria, that can potentiate the physiological effects of SAgs (Stiles et al., 1993). The specific role or interplay of these additional factors in initiating or exacerbating toxic-shock syndrome in women is poorly understood. In addition, this disease is not exclusively linked to the use of tampons. For example, toxic-shock syndrome that is caused by streptococcal pyrogenic exotoxins (SPEs; Reichardt et.al., 1992) sometimes accompanies a severe form of streptococcal pneumonia. Furthermore, chickenpox and other viral infections (Cohen-Abbo and Harper, 1993) may predispose individuals to a toxic-shock syndrome that is caused by SAgs which are produced by secondary streptococcal or staphylococcal infections.

Antibodies reacting with several SAgs are found at low levels in normal human and nonhuman primate sera (Takei. et al., 1993;

Bavari et al., 1995a). Intravenous infusion of normal human γ-globulin containing anti-SAg antibody has been used successfully to treat episodes of the SAg-associated inflammatory disease of children, Kawasaki syndrome (Takei. et al., 1993; Leung et al., 1993). Furthermore, recurrence of toxic-shock syndrome in patients who have recovered from the disease is associated with reduced anti-SAg serum antibody levels (Freedman and Beer, 1991). There is a significant but gradual increase in antibody titers after the acute disease (Stolz et al., 1985).

In a study reported by Vergeront et al. (1983) it was found that 96% of the population examined (Wisconsin residents) had been exposed to TSST-1 by age 30, regardless of gender. The implication of these results was that most individuals received multiple exposures to TSST-1 during their lifetimes and had low but detectable antibody titers to this toxin. However, with the possible exception of studies involving limited patient cohorts, such as those of Kawasaki syndrome, there have been no comprehensive examinations of acquired immunity to other SAgs in humans, especially women in the military.

The specific cellular receptor for SAgs is the major histocompatibility complex (MHC) class II molecule. The complex of MHC class II molecule and SAg initiates a release of toxic levels of cytokines by stimulating a substantial number of T cells. Moreover, SAgs stimulate lymphocytes expressing distinct T-cell antigen receptor Vß subsets. For example, human Vß8-expressing T cells (Irwin et al., 1993) respond preferentially to SEB, while those responding to staphylococcal enterotoxin A (SEA) include Vß5.3, 6.3 and 9.1. SAg can also bind weakly to the T-cell antigen receptor without the MHC molecule (Seth et al., 1994). Thus, each distinct SAg stimulates a fingerprint of specific T-cell subsets during acute exposure, which progresses to a state of specific nonresponsiveness (see below). The acute nonenteric response to SAgs is mediated by activated T cells that release proinflamatory cytokines (Stiles et al., 1994), such as interferon γ, interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α).

Comparison of amino acid sequences suggests that bacterial SAgs fall into groups (Ulrich et al., 1995a) consisting of (1) SEA, SED and SEE, (2) SEB, staphylococcal enterotoxins C1-C3 (SEC1-3), the streptococcal pyrogenic exotoxins A (SPE-A) and C (SPE-C), (3) TSST-1 and (4) the exfoliative toxins (ETA, ETB) and streptococcal pyrogenic exotoxin B (SPE-B), which are the most distant from the others in sequence. Although diverse SAgs appear to have little sequence in common, such as SEB and TSST-1, they exhibit homologous protein folds composed largely of ß strands within two distinct domains (Ulrich et al., 1995a).

All bacterial superantigens use common structural strategies to bind MHC class II receptors, while binding the T-cell antigen receptor in different ways (Ulrich et al. 1995b). A motif consisting of a leucine in a reverse turn is conserved among

bacterial SAgs and may provide the key determinant for binding human MHC class II molecules. The SAg amino acid residues in contact with T-cell antigen receptors are located in regions of high sequence variability, presenting a unique surface for interaction for each SAg. The distinct pattern of stimulated T cell Vß subsets that is observed for each SAg may be a direct result of the heterogeneity in antigen receptor contact residues.

Vaccines produced by genetic inactivation of SAgs have shown promising results in animal trials (Bavari et al. 1995b). This vaccine strategy is based on substitution of critical MHC class II receptor-binding amino acid residues of SAgs that resulted in loss of toxicity, while maintaining antigenicity. The attenuated SAg is processed, recognized as a conventional antigen, and stimulates protective antibodies (Bavari and Ulrich, 1995). Because these vaccines are not superantigenic they can be used as recall antigens to measure T-cell immunity *in vitro,* and therefore provide an accurate indicator of prior exposure.

Stimulation of T cells with SAgs results in rapid cell proliferation. Most of the proliferating T cells are eventually eliminated by a programmed cell death (apoptosis; Nagata and Golstein, 1995). The majority of T cells remaining after the initial proliferative cycle that have not undergone apoptosis do not enter mitosis upon further signaling through the T-cell antigen receptor and are termed anergic (Kawabe and Ochi, 1991). Because the anergy affects multiple T-cell subsets, the induction of neutralizing antibodies by an attenuated SAg vaccine may also be impaired. This is likely to be the reason why TSST-1-specific antibody titers are very slow to appear after the acute phase of toxic-shock syndrome (Stolz et al., 1985), and why they are only present at low levels within the general population. To better define this problem we propose to study the relationship of antibody and T-cell immunity to SEB and the related SAgs as a function of anergy and prior exposure.

BODY

EXPERIMENTAL METHODS

Clinical samples. A total of 100 cc of blood was drawn one time only, after a brief review of donor Medical History by a USAMRIID physician. Donors were active duty military and in good health. Serum and peripheral blood mononuclear cell samples were collected from approximately 50 female and 50 male personnel between the ages of 18 and 35 years of age and in the U.S. Army. Each specimen was assayed immediately after collection and/or cryopreserved.

Antibody assay. Microtiter plates were coated with 1 µg/well of one of the following:SEA, SEB, TSST-1, (recombinant proteins produced in the PI's laboratory), SEC1, SPE-A or SPE-C (Toxin Technologies, Sarasota FL) in 100 µl of PBS (pH 7.4) at 37°C for 2 h. After antigen coating, the wells were blocked with 350 µl of 0.2% casein in PBS for 4 h at 37°C and then washed four times with PBS/tween 20. Human sera was then diluted in PBS with 0.02% casein and 100 µl of each dilution was added to triplicate wells. After each well was washed four times, bound antibody was detected with goat anti-human IgG+M horse radish peroxidase (Sigma Chemical Comp. St Louis, MO) labelled antibody (37°C for 1 h). Color was developed with *O* -phenylenediamine as the chromogen. Absorbance was measured at 490 nm. Mean OD of each experimental group was obtained and data were presented as the inverse of the highest dilution that produced an OD reading four times above the negative control-wells. The antigen or serum were omitted from the control wells.

Neutralization assay. An assay based on LPS potentiation of SE toxicity in mice (Stiles et al., 1993) was used to assess the neutralizing capacity of human sera scoring positive for anti-SEB antibodies. We focused on SEB because different mouse strains must be used to assay the physiological response to each SAg, and the U.S. Army has targeted SEB for vaccine development. For a positive control reference serum, we used pooled sera from rhesus monkeys that were immunized with the proposed SEB vaccine (supplied by Lt. Col. Robert Hunt, U.S. Army) and that survived a challenge with the wild type SEB. Balb/C mice weighing 18-20 g (Harlan Sprague Dawley, Inc., Frederick Cancer Research and Development Center, Frederick, MD) were injected intraperitoneally (i.p.) with 200 µl of PBS containing varying amounts of SEB or SEB preincubated with dilutions of the human sera, followed 4 h later with 75 µg of LPS (200 µl/i.p.). Controls were injected with either SE (10 µg) or LPS (75 µg). Animals were observed for 72 h after the LPS injection. Calculations of LD50 were done by Probit analysis using 95% fiducial limits (SAS Institute Inc., Cary, NC). Because approximately 30 mice are necessary for each dilution of

sera tested, the investigators, where possible, limited the comparisons to a single dilution of serum.

Lymphocyte proliferation. Human peripheral blood mononuclear cells and sera samples were obtained from volunteers on the same day. The mononuclear cells were purified by Ficoll-hypaque (Sigma, St. Louis, MO) buoyant density gradient centrifugation. The mononuclear cells were cryopreserved by suspension in 90% fetal bovine serum (FBS)/10% dimethylsulfoxide (4°C), followed by a slow temperature drop (1°C/min), and final storage in liquid nitrogen. Viabilities of cells after thawing were routinely greater than 99%. The cells were cultured with dilutions of each SAg in RPMI-1640 with 5% FBS for 72 h, and pulsed-labelled for 12 h with 1μCi [^{3}H]-thymidine (Amersham, Arlington Heights, IL). The FBS used for cell cultures contained no detectable anti-SAg antibodies. Cells were harvested onto glass fiber filters, and [^{3}H]-thymidine incorporation into the cellular DNA was measured by a liquid scintillation counter (BetaPlate, Wallac Inc., Gaithersburg, MD).

Detection of T-cell anergy by flow cytometry. Mononuclear cells that were isolated from human donors were cultured with optimal doses of each SAg (e.g. 100 ng/ml for SEA) for 5 days and then incubated in media containing 10% interleukin-2 (Advanced Biotechnologies Inc., Columbia, MD) RPMI-1640 with 5% FBS at 37°C for 5 days. All of the viable cells present in culture at this time point were CD25$^+$ and CD4$^+$. For analysis of T-cell antigen receptor Vß phenotype analysis, the cells were washed twice with phosphate buffered saline and stained with anti-pan T cell receptor, anti-Vß specific TCR monoclonal antibodies (T Cell Sciences, Cambridge, MA) or control isotype matched antibody (45 minutes on ice). Unreacted antibody was removed, and the cells were incubated with a FITC-labelled anti-mouse antibody (Organon Teknika Corp.) on ice for 30 min. The cells were washed and analyzed by flow cytometry on a FACSort (Becton Dikinson & Co.). The experimental phenotypes were compared to similar results obtained with cryopreserved reference lymphocyte cultures known to be nonanergic to the SAgs tested. T cell Vß subsets were scored as a percentage of total cells present and a culture was considered anergic to a specific SAg if the percentage of one or more subsets were significantly reduced compared to the standard. We have compared this flow cytometry method with other techniques, such as polymerase chain reaction amplification of T-cell receptor cDNA, and have found it to more accurately assess the levels of responding T cells (Bavari et al., 1996).

Data analysis. Data obtained from antibody ELISAs, toxin neutralization and T-cell responses were correlated with the gender, race and ethnic origin of the donor, using standard algorithms (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

We addressed the effects of prior environmental exposure to staphylococcal enterotoxin B (SEB) and related *Staphylococcus aureus* and *Streptococcus pyogenes* toxins to the potential immunological responsiveness of women in the military to an SEB vaccine. It was hypothesized that a greater incidence of environmental exposure may occur for women than men and that the residual effects of suppressed toxin-specific immune responses may hinder successful vaccination of the affected women.

Experimental immunology techniques were used to collect epidemiological data concerning the immune responses of enlisted military personnel to the bacterial toxins. The bacterial toxins, such as SEB are often referred to as superantigens, because of their profound effects upon the immune system. A previous environmental exposure to one or more of this group of toxins may produce an inactivation (or anergy) of immune responses, resulting in the potential for vaccination failures that will put immunized troops at risk.

The most common environmental exposure to staphylococcal enterotoxins results in an acute, self-limiting, food poisoning. Toxic-shock syndrome is the most frequent and severe nonenteric disease that is associated with exposure to the toxins from both streptococcal and staphylococcal strains. Toxin-induced anergy is more likely to have occurred with women rather than men, because more than 95% of reported cases of toxic-shock syndrome (linked to these toxins) have occurred in women (Center for Disease Control, 1982). Therefore, this research was initiated because of a lack of information concerning any gender-based differences in levels of circulating antibodies and T cell responses to these bacterial toxins. To investigate the immune status within the vaccine-targeted military population, we measured serum antibody titers and T-cell *in vitro* responses to SEB and related toxins, and also to the recombinant vaccines of SEB, SEA and TSST-1.

A cohort of fifty males and fifty females (ages 19-28) were randomly selected from U.S Army personnel and further studied. No prior medical history was considered other than the requirement that each was healthy and free of obvious disease at the time of donation. There were no provisions for inclusion or exclusion of donors with regard to race or ethnic origin, however data were analyzed for each of these population subgroups. Serum and peripheral blood mononuclear cell samples were collected and assayed immediately or cryopreserved. The presence of antibodies reacting with the following toxins was assessed by ELISA: SEA, SEB, SEC1, TSST-1 and SPE-A. Each individual specimen was scored by measuring the relative levels of specific antibodies in dilutions of sera (titers), and these results were compared to reference positive and negative control sera. Titers (reciprocal of positive sera dilution) for male and female groups ranged from 100-100,000 (Fig. 1). All subjects had serum antibodies reactive with the enterotoxins SEA, SEB, SEC1, and the related toxins TSST-

1 and SPE-A. Antibody titers fell into roughly three groups of high (>5000), medium (>1000) and low (<1000). The mean antibody titers for SEB, TSST-1 and SpeA were greater for females than males (Fig. 2), while for SEA and SEC1 they were similar. The statistical significance of this and related findings are being analyzed. These results may suggest that within the cohort there is a correlation between levels of circulating antibodies reacting with certain bacterial superantigens and gender. The overall frequency of highest antibody titers was also greater for females (Fig. 3A) than males (Fig. 3B) when data was compared by gender. A number of other group matrix analyses have been examined for statistical correlations (results pending).

The serum from individuals that presented anti-SEB titers of high (>5000), medium (>1000) and low (<1000) were used in a human lymphocyte proliferation assay to measure the capacity of the serum to neutralize toxicity. A representative experiment is shown in Figure 4. Serum from representative individuals were mixed with the toxin prior to addition to lymphocyte cultures. The magnitude of inhibition of lymphocyte proliferation (thymidine incorporation) corresponded to the level of anti-SEB present in the sera. An assay based on LPS potentiation of SE toxicity in mice (Stiles et al., 1993) was also used to assess the neutralizing capacity of sera scoring positive for anti-SEB antibodies. BALB/c mice were injected i.p. with 200 µl of PBS containing varying amounts of SEB or SEB preincubated with dilutions of the human sera, followed 4 h later with 75 µg of LPS (200 µl/i.p.). Controls were injected with either SE (10 µg) or LPS (75 µg). Animals were observed for 72 h after the LPS injection. The results are shown in Figure 5. The sera containing high levels of anti-SEB antibody protected mice from a challenge with SEB, while low titer serum was not protective. These data confirmed the relevance of our methods to the *in vivo* immune status of the experimental subjects.

Both T-cell immunity and serum antibody levels are indicators of prior exposure to SAgs and these data are critical because one of the secondary effects of SEB and related toxins is to specifically inactivate T cells, thereby circumventing an appropriate immune response. Short term cultures of lymphocytes from each donor were established and the proliferative responses to SEA, SEB, SEC1, TSST-1, SPE-A and SPE-C were measured. T-cell responses also varied with the toxin and from individual to individual, falling into low, medium and high proliferation ranges (data not shown). We have attempted to search for any statistical correlations between antibody titers and T-cell responses to the toxins within the experimental groups (results pending). A partial representation of antibody titers and T-cell responses data is shown in Figure 6. For each subject, T-cell responses were measured against a wide range of superantigen concentrations, and optimal responses were used for data comparisons. Consistent with the complex nature of immunity to bacterial superantigens, there is no apparent simple correlation between *in vitro* T cell

responses and antibody immunity. In addition the immune response to attenuated, nonsuperantigenic recombinant SEA, SEB vaccines that have been developed in the PIs' laboratory were used to directly evaluate T-cell immunity to the toxins as above. The attenuated TSST-1 could not be produced in sufficient amounts to complete these studies in a timely fashion, and was therefore excluded. The responses to the SEA and SEB vaccines were measured after 6 days in culture and optimal or plateau levels were used for comparison purposes. A representative experiment showing T-cell proliferation to bacterial superantigens and vaccines is shown in Figure 7. Most individuals (all experimental groups) exhibited a moderate or vigorous response to the attenuated vaccines in the concentration range 0.1-50 µg/ml of culture, whereas responses were always evident with >0.001 µg/ml superantigen (3 day cultures). For some individuals there was discordance between circulating antibody levels and *in vitro* proliferative responses to vaccines or superantigens (data not shown). For those cultures that demonstrated significantly reduced proliferative responses for any one or more toxins, as compared to a reference T-cell culture, the frequency of stimulated T-cell receptor Vß subsets which are characteristic for each superantigen was used to score T-cell anergy. Statistical comparisons of results were made between *in vitro* toxin or vaccine response, antibody responses, gender and/or ethnic origin. We have repeated these experimental analyses to confirm our results. Because of the large number of clinical specimens that needed to be analyzed to make valid conclusions, at the time of the submission of this report the final global analyses of data is still in progress. We anticipate the completion of this complex evaluation of the results in the near future.

CONCLUSIONS

Our results represent significant findings pertaining to major factors affecting the health of women in the military, particularly those deployed for military operations, and of women within the general civilian population. All individuals examined, regardless of gender, had evidence of prior exposure to one or more *Staphylococcus aureus* or *Streptococcus pyogenes* toxin. Anergy of T-cell and/or antibody immunity was observed for some individuals. Distinct gender-related differences in levels of specific anti-toxin immune responses were suggested, as judged by serum antibody titers and *in vitro* lymphocyte responses. Insufficient data are available at present to prove causal relationships. We conclude that the subject population will benefit from immunization with an SEB vaccine to boost low or moderate pre-existing serum antibody titers, but that some vaccine recipients, especially women, may not adequately develop high titers of circulating antibodies because of potential preexisting anergy. Monitoring antibody and T-cell responses is indicated as an appropriate precaution to assure the successful vaccination of military women specifically immunized for protection against exposure to SEB and related bacterial toxins.

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APPENDIX I

FIGURE LEGENDS

Figure 1. Serum antibodies reacting with staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C1 (SEC1), toxic shock syndrome toxin-1 (TSST-1) and streptococcal pyrogenic exotoxin A (SpeA). Each individual specimen was scored by measuring the relative levels of specific antibodies in dilutions of sera (titers). Titers (reciprocal of positive sera dilution) for male and female groups ranged from 100-100,000, and fell into roughly three groups of high (>5000), medium (>1000) and low (<1000). SEM<5%, in triplicates.

Figure 2. A comparison of antibody titers (means of data) by gender for staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C1 (SEC1), toxic shock syndrome toxin-1 (TSST-1) and streptococcal pyrogenic exotoxin A (SpeA). SEM<5%, in triplicates.

Figure 3. Frequency of antibody levels (titers) reactive with staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C1 (SEC1), toxic shock syndrome toxin-1 (TSST-1) and streptococcal pyrogenic exotoxin A (SpeA), compared by gender. Females (A); males (B). SEM<5%, in triplicates.

Figure 4. Inhibition of human lymphocyte proliferation ([3H]thymidine incorporation) correlates with the level of anti-SEB present in sera. The serum from individuals that presented anti-SEB titers of high (>5000), medium (>1000), low (<1000) or negative were used in a human lymphocyte proliferation assay to measure the capacity of the serum to neutralize toxicity. Serum from representative individuals were mixed with the toxin prior to addition to lymphocytes, and cultures were maintained for a total of 3 days. SEM<5%, in triplicates.

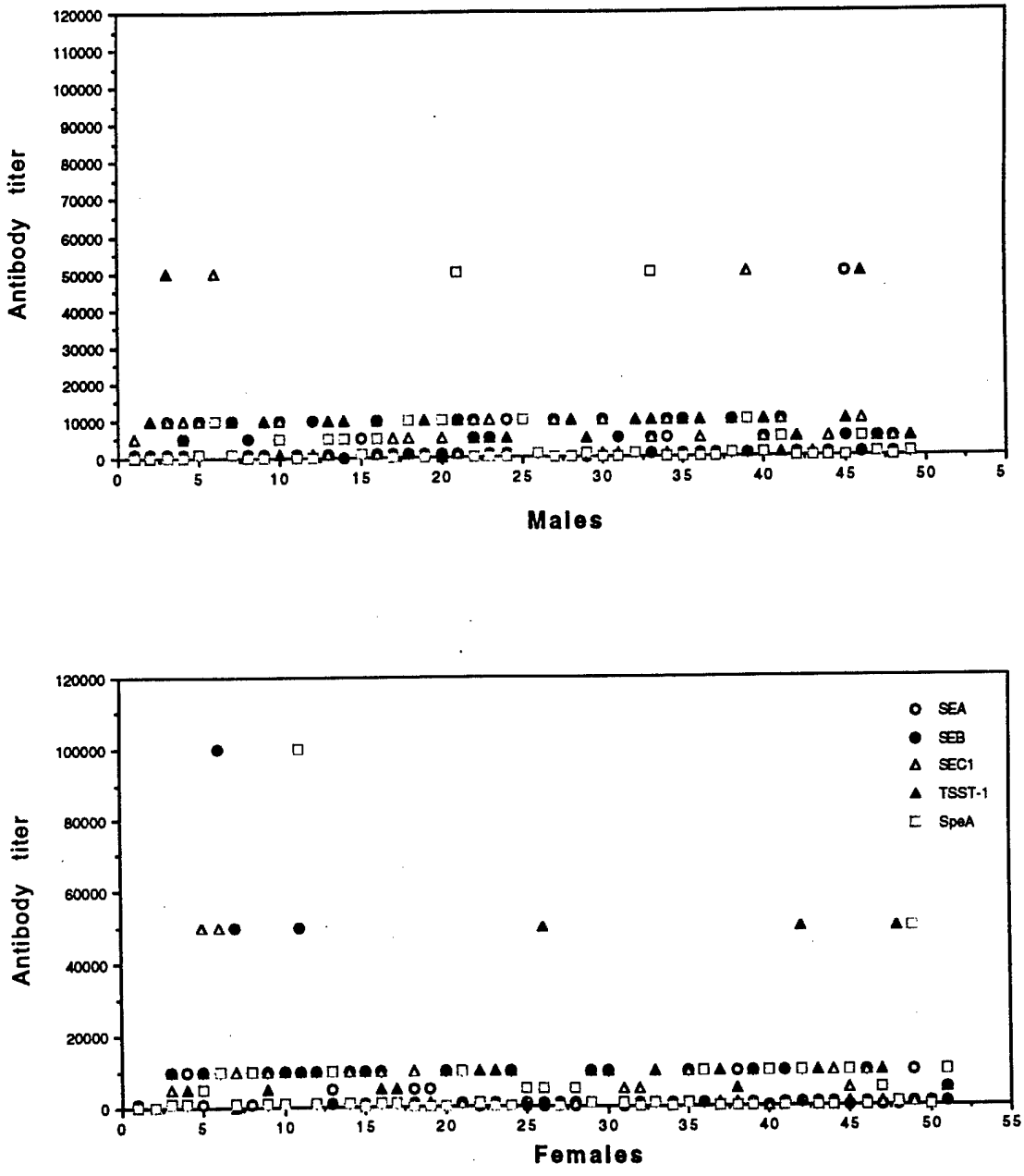
Figure 5. Human sera containing high levels of anti-SEB antibody protected mice from a challenge with SEB, while low titer sera were not protective. An assay based on LPS potentiation of SE toxicity in mice (Stiles et al., 1993) was also used to assess the neutralizing capacity of sera scoring positive for anti-SEB antibodies. BALB/c mice were injected i.p. with 200 µl of PBS containing varying amounts of SEB or SEB preincubated with dilutions of the human sera, followed 4 h later with 75 µg of LPS (200 µl/i.p.). Controls were injected with either SE (10 µg) or LPS (75 µg). Animals were observed for 72 h after the LPS injection. All control animals survived the challenge with SEB.

Figure 6. A comparison, by gender, between antibody titers and T-cell responses to staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C1 (SEC1), toxic shock syndrome toxin-1 (TSST-1) and streptococcal pyrogenic exotoxin A (SpeA). For each subject, T-cell responses were measured against a wide range of superantigen concentrations, and optimal responses were used for data comparisons. SEM<5%, in triplicates.

Figure 7. T-cell responses to attenuated, nonsuperantigenic recombinant staphylococcal enterotoxin A and B (SEA, SEB) vaccines. The responses to the vaccines were measured after 6 days in culture and optimal or plateau levels were used for comparison purposes. Most individuals (all experimental groups) exhibited a moderate or vigorous response to the attenuated vaccines in the concentration range 0.1-50 µg/ml of culture, whereas responses were always evident with >0.001 µg/ml superantigen (3 day cultures). A representative experiment is shown. The *in vitro* response to the vaccines reflects prior T-cell immunity to the toxins SEA and SEB. SEM<5%, in triplicates.

APPENDIX II

FIGURES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100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0
5
10
15
20
25
30
35
40
45
50
5
Males
Antibody titer
120000
100000
80000
60000
40000
20000
0
0
5
10
15
20
25
30
35
40
45
50
55
Females
SEA
SEB
SEC1
TSST-1
SpeA

Figure 1.

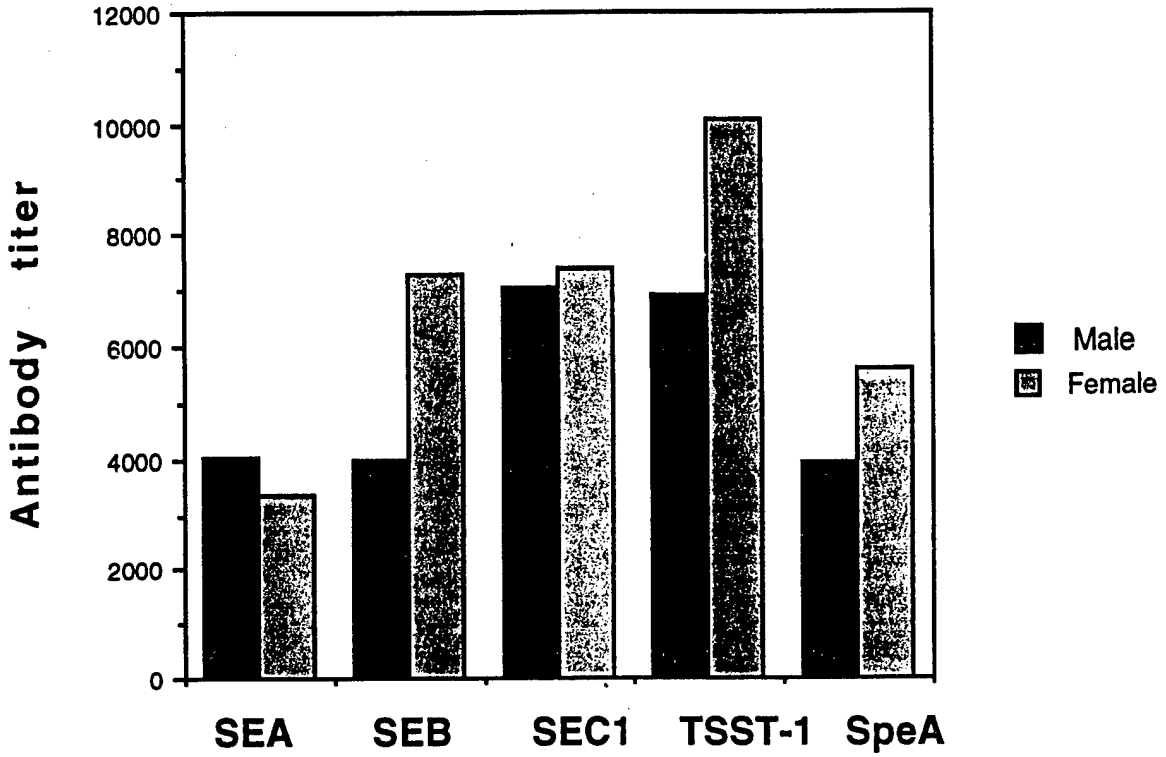
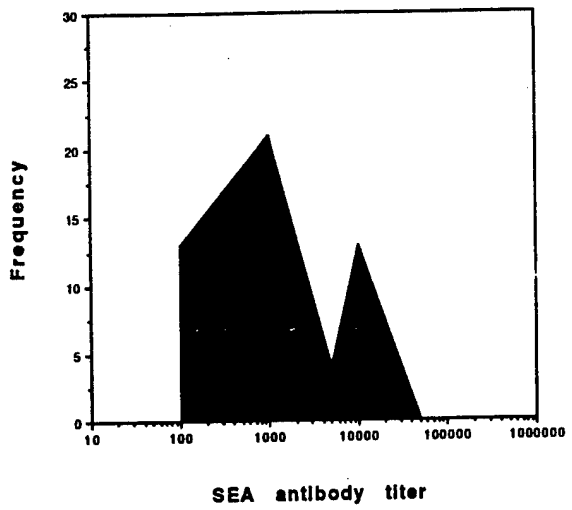
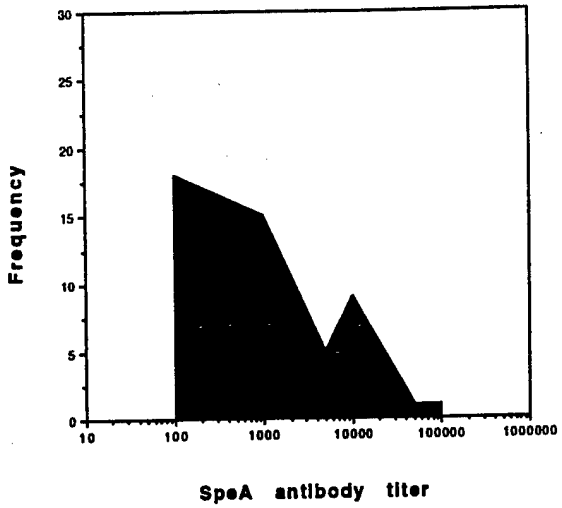
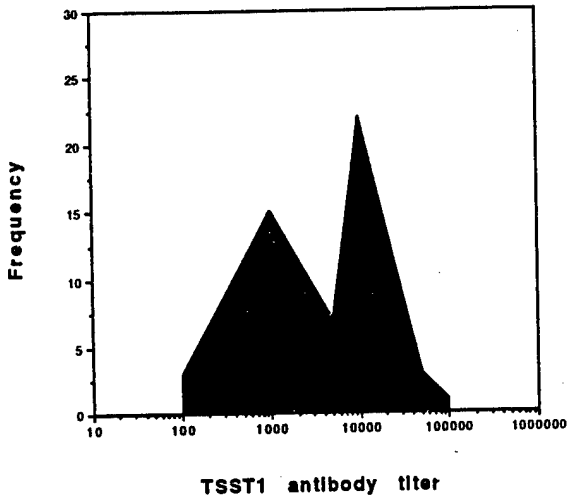

12000
10000
8000
6000
4000
2000
0
Antibody titer
SEA
SEB
SEC1
TSST-1
SpeA
Male
Female

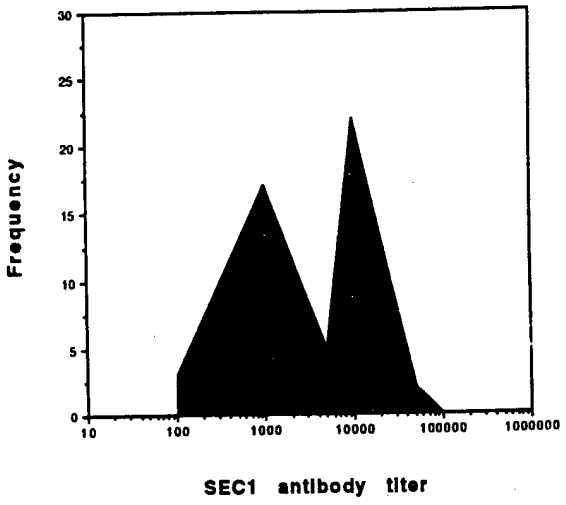
Figure 2.

Figure 3A.


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
1000000
SEA antibody titer


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
1000000
SpeA antibody titer


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
1000000
TSST1 antibody titer


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
1000000
SEC1 antibody titer

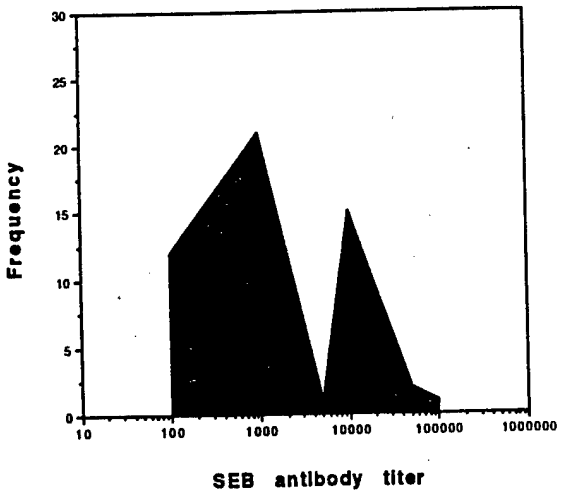
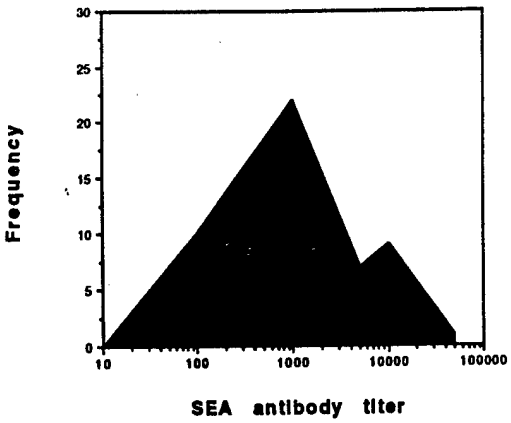
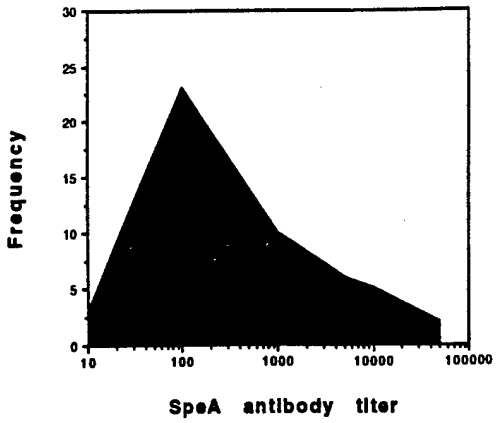
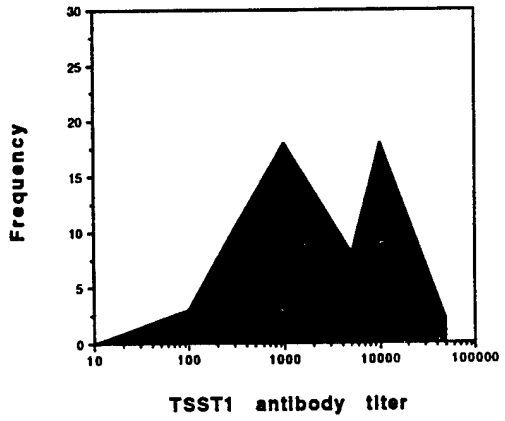
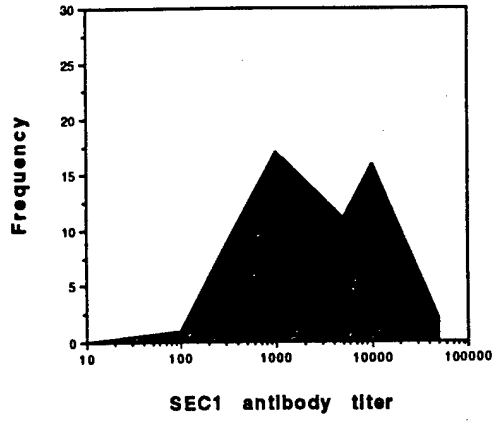

Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
1000000
SEB antibody titer

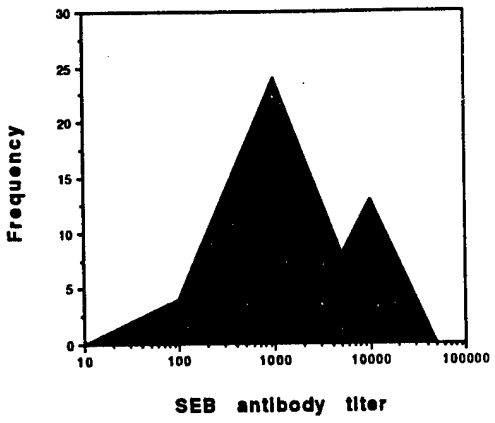
Figure 3B.


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
SEA antibody titer


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
SpeA antibody titer


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
TSST1 antibody titer


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
SEC1 antibody titer


Frequency
30
25
20
15
10
5
0
10
100
1000
10000
100000
SEB antibody titer

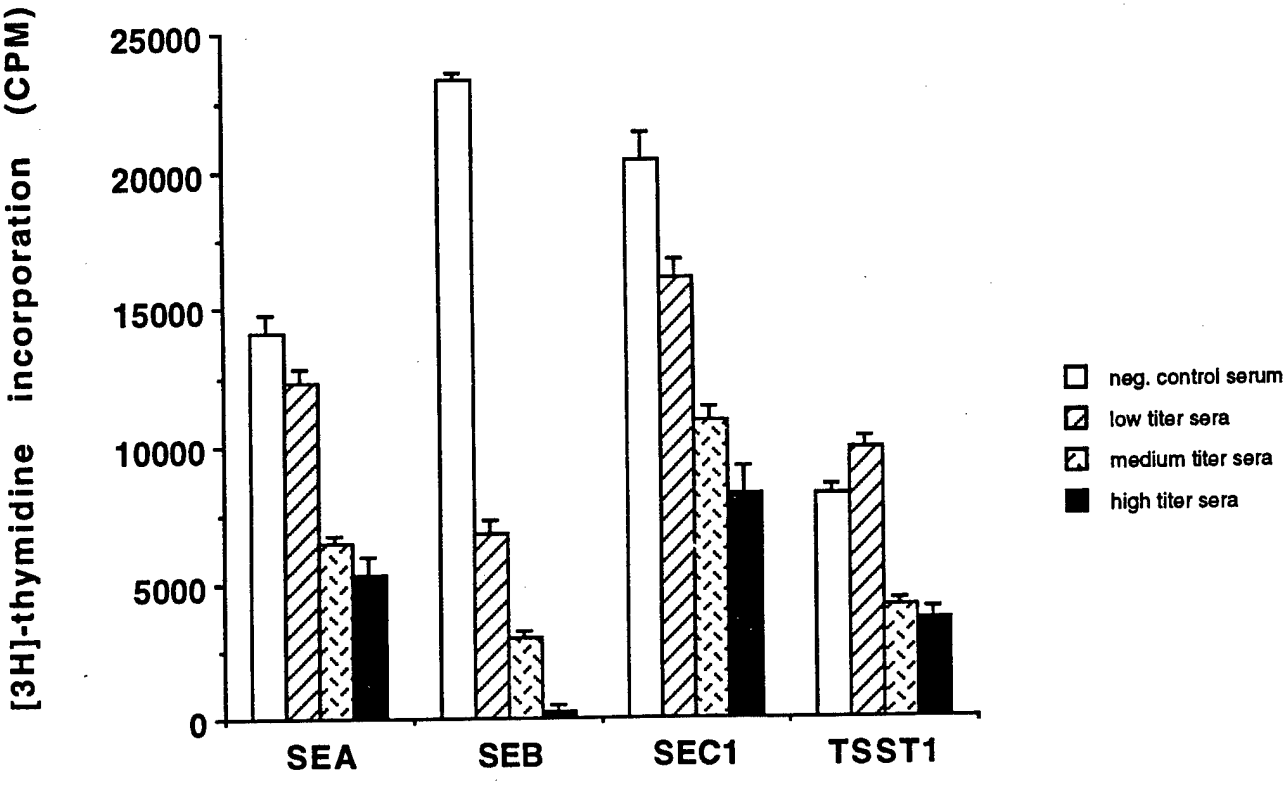

[3H]-thymidine incorporation (CPM)
25000
20000
15000
10000
5000
0
SEA
SEB
SEC1
TSST1
neg. control serum
low titer sera
medium titer sera
high titer sera

Figure 4.

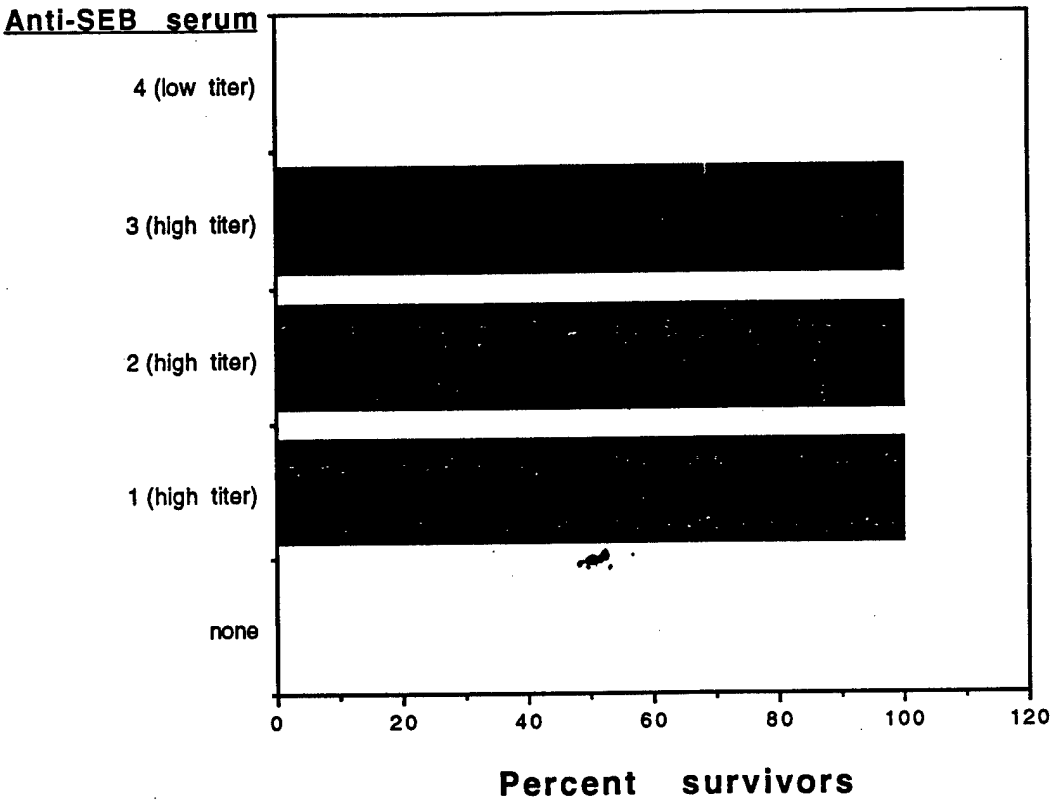

Anti-SEB serum
4 (low titer)
3 (high titer)
2 (high titer)
1 (high titer)
none
0
20
40
60
80
100
120
Percent survivors

Figure 5.

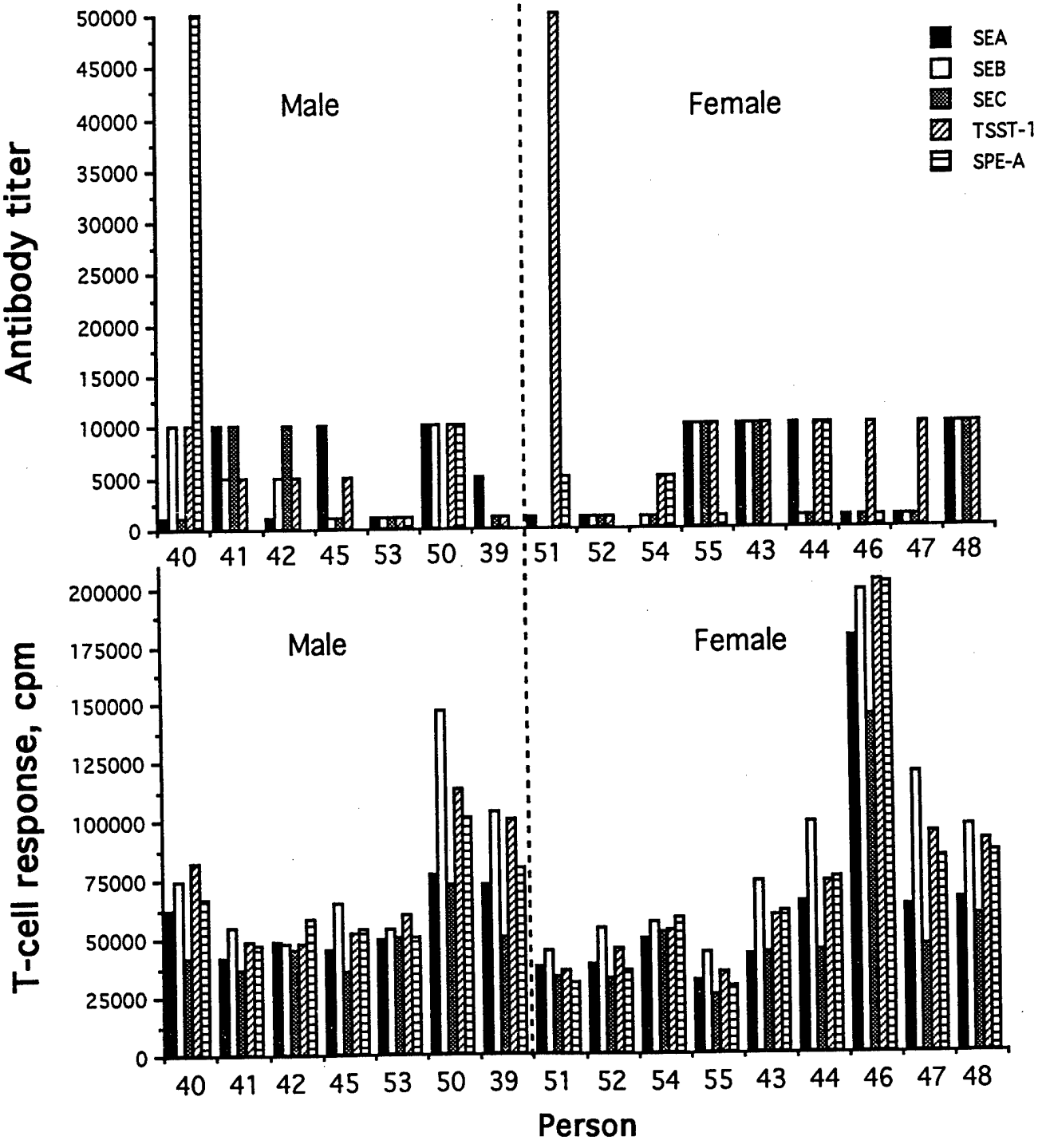

Antibody titer
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Male
Female
SEA
SEB
SEC
TSST-1
SPE-A
40 41 42 45 53 50 39 51 52 54 55 43 44 46 47 48
T-cell response, cpm
200000
175000
150000
125000
100000
75000
50000
25000
0
Male
Female
40 41 42 45 53 50 39 51 52 54 55 43 44 46 47 48
Person

Figure 6.

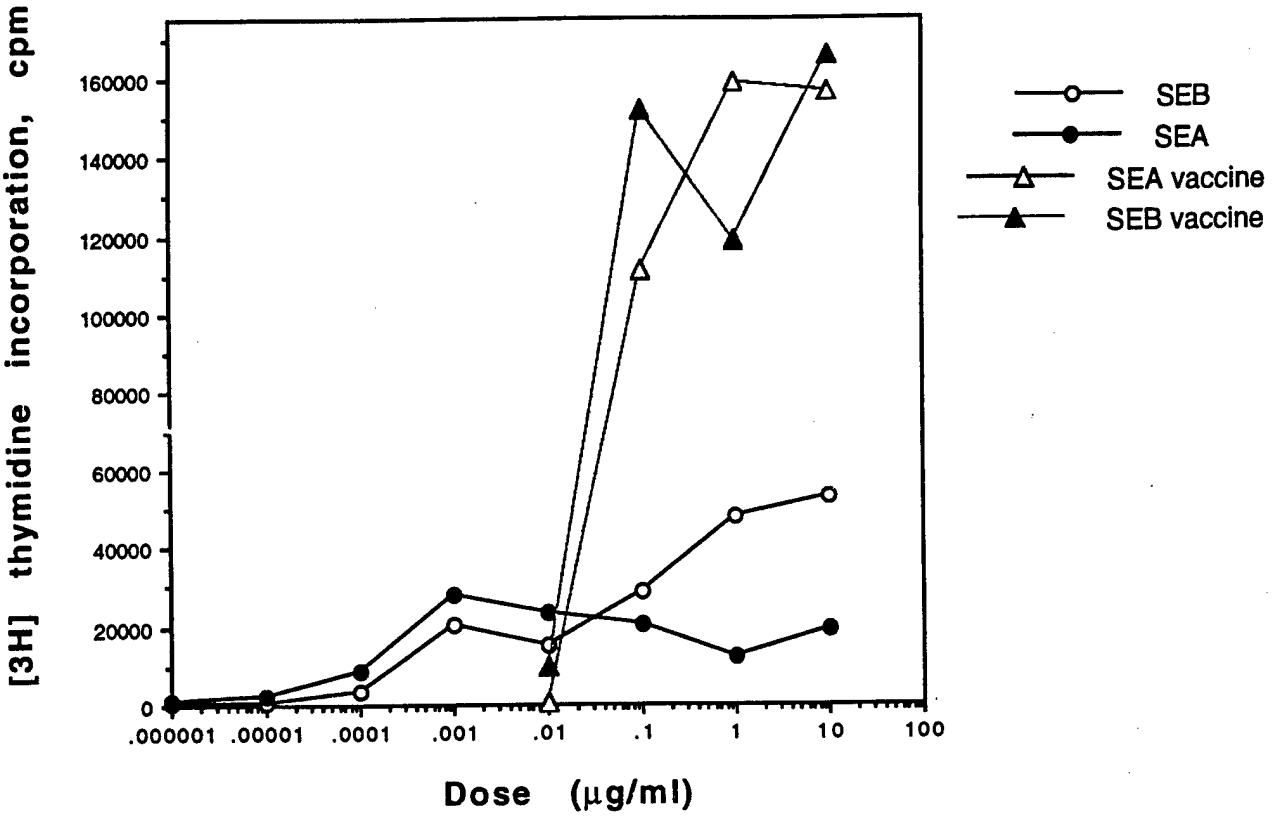

[3H] thymidine incorporation, cpm
160000
140000
120000
100000
80000
60000
40000
20000
0
.000001
.00001
.0001
.001
.01
.1
1
10
100
Dose (µg/ml)
SEB
SEA
SEA vaccine
SEB vaccine

Figure 7.

APPENDIX III

BIBLIOGRAPHY OF PUBLICATIONS FROM THE NEGOTIATED EFFORT

Ulrich, R.G., Dyas, B., Saunders, S. and Bavari, S. Evidence of innate T-cell anergy in women resulting from environmental exposure to bacterial superantigens. Manuscript in preparation

APPENDIX IV

LIST OF PERSONNEL RECEIVING PAY FROM THE NEGOTIATED EFFORT

TITLE	NAME	EFFORT
Principal Investigator:	Robert G. Ulrich	20%
Coinvestigator:	Sina Bavari	10%
Graduate student:	Sheri Saunders	100%
Research Technician:	Beverly Dyas	20%